Vascular function in chronic end-organ damage

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Chapter 8

Effects of chronic EGFR inhibition on vascular contractile function in a rat model of kidney I/R injury

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Abstract

Introduction: Acute renal failure (ARF) caused by ischemia/reperfusion (I/R) injury significantly reduces the survival rate in intensive care unit patients. The epidermal growth factor receptor (EGFR) signaling is shown to be activated after kidney I/R and a local increase in the kidney after ARF may precede a systemic upregulation in the growth factor signaling. We recently showed the transactivation of EGFR to mediate in part phenylephrine (PE) induced contraction of isolated rat aorta. Therefore, we aimed to explore the effects of chronic PKI-166 (EGFR kinase inhibitor) treatment on PE mediated aorta contraction in a rat model of kidney I/R injury.

Methods: Sham or I/R operations were performed in 12 weeks old male Wistar rats. Starting 1 hour before the operation rats received either orally PKI-166 (100 mg/kg/day) or vehicle treatment. Thoracic aorta was isolated 4 days after sham operation or 14 days after I/R injury. Plasma creatinine, renal pre-fibrotic changes and i*n vitro* aortic contractile responses to PE and KCl were investigated.

Results: Plasma creatinine levels were significantly higher in untreated and PKI-166 treated I/R animals compared to sham animals. Chronic PKI-166 treatment did not change PE dose-response curves in sham, but caused a significant reduction of E_{max} in the I/R group. Acute EGFR inhibition in the rings with AG1478 attenuated PE dose-response curves in all groups. Chronic EGFR inhibition did not change KCl mediated rat aorta contraction.

Conclusions: Our data suggest that the non-EGFR dependent component of PE mediated aorta contraction is attenuated by EGFR blockade after kidney I/R injury in rats.

Introduction

Severe kidney hypoperfusion causes acute kidney injury which may lead to ischemic acute renal failure (ARF). ARF is common in intensive care unit patients and significantly reduces the survival rate in this patient population.[1] In addition to the injury induced by ischemia *per se*, reperfusion of the kidneys causes additional damage, which is mediated mostly by reactive oxygen species (ROS) formation[2] and inflammation.[3,4] This is particularly important in kidney transplantation because ischemia-reperfusion (I/R) injury affects both its short-term[5] as well as long-term[6] outcome. Therefore, therapy targeting the underlying pathophysiology of I/R injury may improve mortality and morbidity in ARF.[7] Unfortunately, however, the molecular changes involved in the onset of I/R has not been fully defined yet.

The repair process in ARF has been shown to involve a number of growth factors such as epidermal growth factor (EGF), heparin binding-epidermal growth factor (HB-EGF), and transforming growth factor-α (TGF-α) produced in the kidney, which function as autocrine or paracrine regulators of the repair mechanism.[8,9] It is known that activation of the epidermal growth factor receptor (EGFR) leads to a cascade of pro-inflamatory/pro-fibrogenic genes and proteins such as interleukins, growth factors and chemokines. In a previous study, Kwon et al showed that I/R induces activation of extracellular signal-regulated kinase (ERK) and Akt signaling pathways through the ROS-dependent EGFR cascade.[10]

Given the importance of growth factors in kidney I/R injury, it is unclear whether their local increase in the kidney after ARF precedes a systemic upregulation in the growth factor signaling. We recently showed the transactivation of EGFR to mediate in part the phenylephrine (PE) induced contraction of isolated rat aorta in healthy animals.[11] Thus, a possible systemic activation of EGFR after kidney I/R injury may alter PE mediated aorta contractility. To investigate this, the effect of chronic EGFR inhibitor (PKI-166) treatment on aortic vascular contractile function was investigated in a rat model of kidney I/R injury. Further, systemic EGFR inhibition may protect the kidney against injury by preventing the activation of pro-inflammatory genes and

eventually fibrosis. Therefore, the renal effects of chronic PKI-166 treatment were also explored.

Materials and methods

Animals

Experiments were performed on 12 weeks old (280-300 g) male Wistar rats. Animals were housed under standard conditions of temperature (21-24°C), humidity (40-60%) and 12 h light:dark cycle at the animal facilities of the University of Groningen. All animals had free access to food (standard rat chow; Hope Farms, Woerden, The Netherlands) and drinking water throughout the study. All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Committee for Animal Experiments of the University of Groningen.

Ischemia/reperfusion protocol

Rats were randomly divided into 4 groups (n=5-6 rats per group), and anesthetized with 2% isoflurane. A left-flank incision was made, the left renal vessels were dissected and clamped with atraumatic clips for 45 min (ischemic time). After removing the clips, reperfusion of the kidney was confirmed visually, abdomen and skin layers were sutured with 4.0 stitches. Sham-operated rats underwent dissection of the left renal pedicle without clamping. Ischemia/reperfusion (I/R) animals received either EGFR kinase inhibitor treatment (PKI-166, 100 mg/kg/day, orally by gavage; I/R+PKI-166 group) or an equal volume of vehicle (orally by gavage; I/R+Vehicle group) for 14 days starting one hour before the operation. Sham animals received either PKI-166 treatment (100 mg/kg/day, orally by gavage; Sham+PKI-166 group) or equal volume of vehicle (orally by gavage; Sham+Vehicle group) for 4 days starting one hour before the operation. At the end of the reperfusion period, rats were anesthetized; the clipped kidney was perfused with saline and harvested. A mid-coronal kidney slice was fixed in 4% paraformaldehyde, processed for paraffin embedding and used for immunohistochemical analysis. Thoracic aorta was isolated for the measurement of vascular contractile function.

Tissue preparation and tension studies in rat aortic rings

Thoracic aorta segments (approximately 2 mm) were isolated, and cleared of fat and connective tissue. Endothelium denudation was established by gentle rubbing of the intimal surface with a paper clip. Rings were mounted in a 15 ml organ bath with Krebs solution (pH 7.5) containing (in mmol/L): NaCl (120.4), KCl (5.9), $CaCl_2$ (2.5), $MgCl_2$ (1.2), NaH_2PO_4 (1.2), glucose (11.5), $NaHCO_3$ (25.0) which was kept at 37 °C and continuously bubbled with 95% O_2 and 5% CO_2. Prior to isotonic measurements of vascular contractility, rings were allowed to equilibrate for 40 min. To test for viability of smooth muscle cells, arteries were preconstricted with KCl (60 mM) two times. After wash out and another 30 min of stabilization endothelium denudation was confirmed by the absence of dilative response to ACh (30 μM) following a submaximal pre-constriction with KCl (40 mM). After wash out and another 30 min of stabilization, rings were incubated either with EGFR kinase inhibitor AG1478 (10 μM) or dimethyl sulphoxide (DMSO, 0.5% final concentration; vehicle) for 20 min, and subsequently aorta contractility was measured as contraction response to cumulative doses of PE (1 nM –10 μM). Finally, KCl (60 mM) was added to the organ baths.

Histomorphological determination of kidney injury

Paraffin embedded kidney sections were cut in 3 μm thick slices and stained with α-smooth muscle actin (SMA), a marker of pre-fibrosis (mouse monoclonal antibody, clone 1A4, Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). Interstitial SMA was measured by using computer-assisted morphometry. Thirty fields were randomly selected, evaluated at ×20 magnification by measuring the intensity of the staining per field, as described previously.[12]

Solutions and drugs

The dose of PKI-166 (100 mg/kg/day) was calculated for individual rats and first dissolved in 1 ml DMSO (Serva electrophoresis GmbH, Heidelberg, Germany)+50 μl Tween80 (Fluka AG, Buchs, Switzerland) solution and then

diluted in 10% DMSO with water. All compounds for Krebs solution, and AG1478 were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). PKI-166 was kindly provided by Dr. Giorgio Caravatti (Novartis Pharma AG, Basel, Switzerland).

Statistical analysis and calculations

Data are expressed as mean±SEM; *n* values represent the number of investigated rats. SPSS 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA) software was used for statistical analysis. Body weights and creatinine values were compared by Oneway ANOVA followed by Bonferroni *post hoc* test for multiple comparisons. Concentration-response curves from aortic rings were compared by ANOVA for repeated measures followed by Bonferroni *post hoc* test for multiple comparisons. Differences were considered significant at $P<0.05$ (two-tailed).

Results

Phenylephrine mediated vascular contractile function after kidney I/R injury

The cumulative dose-responses to PE in isolated endothelium-denuded aortic rings obtained from sham and I/R groups are shown in figure 1. The characteristics of vasoreactivity of these aorta rings are presented in table 1. Chronic treatment of sham animals with PKI-166 did not affect PE mediated aorta contractions, or the inhibition of this contraction by AG1478. (figure 1). Whereas I/R did not affect PE contractility of untreated rats, chronic treatment with PKI-166 caused a marked decrease in PE mediated contraction in I/R animals (figure 1). Remarkably, AG1478 inhibited the PE response of both untreated and treated animals to a similar extent (figure 1, table 1). Collectively, these data suggest chronic inhibition of EGFR after kidney I/R injury attenuates the non-EGFR dependent component of α_1-AR mediated aorta contraction.

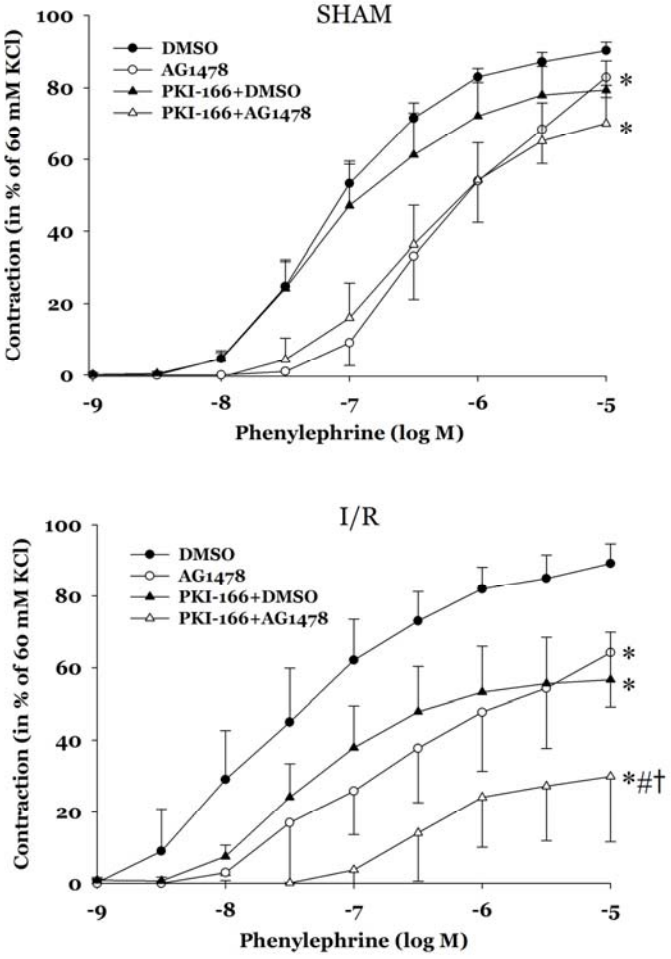

Figure 1. Concentration-response curves to phenylephrine in sham (upper panel) and kidney ischemia/reperfusion injury (I/R; lower panel) rats. DMSO: Vehicle control for untreated rats, AG1478: Acute EGFR inhibition in untreated rats, PKI-166+DMSO: Vehicle control for PKI-166 treated rats, PKI-166+AG1478: Acute EGFR inhibition in PKI-166 treated rats. Data are expressed as means±SEM. n=5-6 rats per condition. *P<0.05 versus DMSO (Vehicle control), #P<0.05 versus AG1478, †P<0.05 versus PKI-166+DMSO.

Table 1. *Characteristics of vasoreactivity of thoracic aorta rings isolated from experimental groups.*

	SHAM			
	Untreated		PKI-166 treated	
	DMSO	AG1478	DMSO	AG1478
$pD2$	7.2±0.1	6.3±0.2*	7.0±0.2	6.5±0.2
E_{max}	90.4±2.4	83.0±5.6	79.5±8.1	70.1±10.7
AUC	207±22	100±22*	164±26	105±25*
	I/R			
	Untreated		PKI-166 treated	
	DMSO	AG1478	DMSO	AG1478
$pD2$	7.7±0.3	7.0±0.2	7.2±0.2	7.4±0.4
E_{max}	89.2±5.4	64.2±15.0*	56.7±13.2*	29.9±18.3*
AUC	221±36	155.2±25*	119±36*	69.8±37*

Data are given as means±SEM, n=5-6 rats per group; pD_2: negative logarithm of molar concentration of phenylephrine causing half maximal response (EC_{50}), E_{max}: maximal contraction to phenylephrine in % of KCl contraction, AUC: area under the curve. *P<0.05 versus Untreated DMSO.

KCl mediated vascular contractile function after kidney I/R injury

To investigate whether the observed decrease in PE mediated contractility of in PKI-166 treated I/R rats represents a general decrease in contractile capacity, contractile responses to 60 mM KCl were obtained in endothelium-denuded aortic rings from sham and I/R groups (figure 2). KCl induced aorta contractions were similar both in vehicle and PKI-166 treated sham (figure 2, upper panel) and I/R (figure 2, lower panel) rats. Acute EGFR inhibition by AG1478 caused a non-significant attenuation in KCl induced aorta contractions in sham and I/R rats, both to a similar extent. Therefore, these data demonstrate

that chronic *in vivo* and acute *in vitro* EGFR inhibition, or kidney I/R injury does not affect KCl induced aorta contractions.

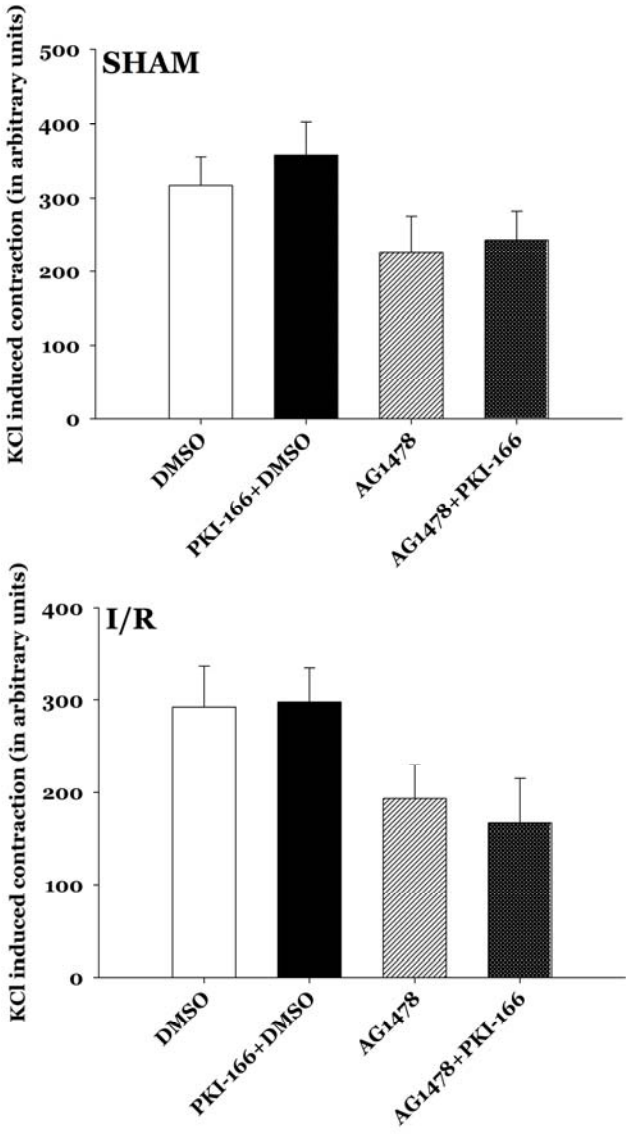

Figure 2. Absolute contractile responses to 60 mM KCl in isolated endothelium-denuded aortic rings obtained from sham (upper panel) and kidney ischemia/reperfusion injury (I/R; lower panel) rats. DMSO: Vehicle control for untreated rats, AG1478: Acute EGFR inhibition in untreated rats, PKI-166+DMSO: Vehicle control for PKI-166 treated rats, PKI-166+AG1478: Acute EGFR inhibition in PKI-166 treated rats. Data are expressed as means±SEM. *n*=5-6 rats per condition.

Table 2. *The effect of kidney I/R injury on plasma creatinine and α-smooth muscle actin (SMA) staining score as a marker of pre-fibrosis.*

	Sham		Ischemia/Reperfusion	
	Untreated	**PKI-166**	**Untreated**	**PKI-166**
Plasma Creatinine (μM)	23.1±1.1	23.0±1.4	34.1±2.6*	32.4±2.8*
SMA score	1.1±0.2	1.3±0.2	10.9±0.7*	11.6±2.4*

Data are given as means±SEM, n=5-6 rats per group. *P<0.05 versus corresponding sham groups.

The renal effects PKI-166 treatment after kidney I/R injury

Pre-fibrotic changes after kidney I/R injury were evaluated by measuring interstitial SMA intensity and data are presented in table 2. SMA staining significantly increased 14 days after I/R in both I/R groups (P<0.05 versus corresponding sham groups). Also, plasma creatinine levels were increased both in I/R+Vehicle (P<0.05 versus Sham+Vehicle) and I/R+PKI-166 groups (P<0.05 versus Sham+PKI-166), validating the I/R model (Table 2). Therefore, these data collectively demonstrate that PKI-166 treatment at a dose of 100 mg/kg/day did not attenuate I/R injury in the kidneys.

Discussion

Our data in sham rats further extend our previous findings on the role of EGFR in PE mediated isolated rat aorta contractility by showing that chronic *in vivo* EGFR inhibition did not influence PE mediated contractions in contrast to acute *in vitro* EGFR inhibition. Therefore, EGFR transactivation in α_1-ARs mediated large artery contractility is most likely activated for a transient period with rapid kinetics[13] in rats. However, chronic inhibition of EGFR after kidney I/R injury seems to attenuate the non-EGFR dependent component of α_1-AR mediated aorta contraction. Also, chronic EGFR inhibition, at least at the dose

used in this study, was unsuccessful to prevent pre-fibrotic changes or the decline in renal function following kidney I/R injury.

The kidney is one of the highly specialized organs that can resist the detrimental effects of moderate ischemia. After severe I/R the renal response becomes insufficient leading to severe inflammation and eventually ARF. It is known that both ischemia and reperfusion involves cellular injury,[14-16] oxidative stress,[2,17] and inflamation.[3,14] Recently, ERK and Akt signaling pathways have been implicated after I/R through the ROS-dependent EGFR cascade.[10] However, the role of EGFR signaling involved in I/R has not been fully defined yet.

EGFR, also named ErbB1, is classified under the ErbB receptor family, and is known to exert several effects in kidney. On the one hand, constitutive ErbB receptor signaling is known to be required for normal development. On the other hand, inhibition of the excess ErbB activity to the basal level is thought to be beneficial in experimental renal fibrotic conditions.[18] More likely EGFR signaling has bidirectional effects in I/R states. Our experimental approach therefore provided a suitable model of kidney I/R with fibrosis to test whether EGFR inhibition has a therapeutic potential. However, we could not provide evidence for attenuation of kidney injury in PKI-166 treated I/R animals. Nevertheless, lack of improvement in our study cannot rule out the involvement of EGFR signaling in kidney injury, because, as mentioned above, the level of EGFR activity plays a crucial role and influences the long-term outcome after initiation of fibrosis. Therefore, both the dose, and the duration of the treatment may need optimization.

To question whether the local increase in the kidney after ARF precedes a systemic upregulation of growth factor signaling, we evaluated the involvement of EGFR signaling in PE mediated aorta constriction. Our recent work reveals a role for EGFR transactivation in PE induced constriction of isolated rat aorta, as acute inhibition of EGFR by AG1478 concentration dependently attenuates PE induced aorta contractions. In contrast to our findings with acute EGFR inhibition, chronic PKI-166 treatment did not modify α_1-AR mediated contractions in sham animals. However, in I/R rats, chronic inhibition of EGFR by PKI-166 significantly attenuated PE induced aorta contraction. Moreover, additional acute

inhibition of EGFR by AG1478 antagonized the PE mediated contraction in I/R rats, demonstrating that α_1-ARs mediated signaling through EGFR is intact in I/R animals treated chronically with an EGFR inhibitor. Since KCl induced aortic contractions were comparable in sham and I/R groups, either treated or untreated with PKI-166, an alteration in Ca^{2+} sensitivity seems unlikely to explain the effect of PKI-166 in I/R injury. Therefore, these results suggest that chronic inhibition of EGFR after kidney I/R injury attenuates the non-EGFR dependent component of α_1-AR mediated aorta contraction. A possible mechanism of the decrease of this component of constriction may root in an excessive stimulation of vascular α_1-AR after kidney I/R injury, which would only affect the non-EGFR dependent signaling route due to the chronic blockade of EGFR dependent route. In line with this hypothesis, an increase in the levels of norepinephrine in the circulation was observed after kidney I/R injury[19] due to an activation of afferent renal nerve activity and a reflex activation of the efferent renal sympathetic nerve.[20] Further support for α_1-AR overstimulation following renal I/R injury is derived from a study showing renoprotective effects of systemic, pre-ischemic prazosin treatment, supporting that excessive release of norepinephrine is involved in the pathogenesis of ischemic acute renal failure, and its consequences.[19] Thus, chronic EGFR inhibition by PKI-166 may indeed be beneficial by attenuating vascular sensitivity to the agonists of α_1-AR after I/R injury. This assumption needs to be further explored in future studies.

In conclusion, our data suggest that the non-EGFR dependent component of PE mediated aorta contraction is attenuated by EGFR blockade after kidney I/R injury in rats. Chronic EGFR inhibition, at least at the dose used in this study, seems to be unsuccessful in prevention of pre-fibrotic changes in kidney I/R injury.

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